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Sanitizer Efficacy against *Escherichia coli* O157:H7 Biofilms on Inadequately Cleaned Meat-contact Surface Materials

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ABSTRACT

This study evaluated the efficacy of various sanitizers against *Escherichia coli* O157:H7 cells in biofilms formed on surface materials used in beef fabrication facilities. Coupons (2 × 5 cm) of stainless steel, acetal, and high-density polyethylene were inoculated (3–4 log CFU/cm^2) with rifampicin-resistant *E. coli* O157:H7 (6-strain mixture) and incubated at 15°C in an unsterilized beef fat-lean tissue homogenate (pH 5.66). After 3 days of incubation, attached cells were challenged (for 1 or 10 min) by submerging coupons in minimum and maximum recommended concentrations of each of seven sanitizing solutions or distilled water (control). Sanitizer treatments reduced *E. coli* O157:H7 on coupons by 0.0 to 2.2 log CFU/cm^2, and treatment efficacy decreased in the order acidified sodium chlorite > peroxyacetic acid > potassium peroxymonosulfate/sodium chloride = peroxyacetic acid/octanoic acid mixture (PA/OA) > cetylpyridinium chloride > quaternary ammonium chloride compound mixture (QACC) = sodium hypochlorite (SH) = water control. Pathogen reductions generally increased as sanitizer concentration and exposure time increased. The influence of biofilm age (0, 3 and 7 days incubation at 15°C) on sanitizer (SH, QACC and PA/OA) efficacy was evaluated in a separate experiment; results showed that *E. coli* O157:H7 biofilm cells became less sensitive to most sanitizer treatments as biofilm age increased. Surface material did not ($P \geq 0.05$) influence the fate of biofilm cells during sanitizing treatments. While no sanitizer consistently reduced pathogen populations by more than 2.2 log cycles on soiled surfaces, approved concentrations of acidified sodium chlorite and peroxyacetic acid-based sanitizers may be more effective than other sanitizers against *E. coli* O157:H7 on inadequately cleaned surfaces.

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INTRODUCTION

Pathogens, including *Escherichia coli* O157:H7, have been recovered from beef fabrication-floor conveyor belts during both pre-operational and mid-shift inspections *(18)*. Other research groups have recovered viable bacteria from conveyor belts and carcass breaking/cutting surfaces *(5, 10, 26)*, and inadequately sanitized food-contact surfaces have been implicated as sources of contamination in outbreaks of foodborne illness *(16)*. *E. coli* O157:H7 has also been shown to attach to stainless steel *(17, 21, 22)*. Wilks et al. *(29)* found that *E. coli* O157 survived for 28 days at 4°C on stainless steel, while copper and copper alloys were bactericidal. Even so, the functionality of stainless steel has resulted in its extensive use as a building material for food processing equipment and facilities. Other materials commonly used to construct food processing equipment include acetal, polypropylene and high-density polyethylene (HDPE). While not all aspects of biofilm formation and persistence are clear, it is apparent that: (i) biofilms can form on meat-contact surface materials, including those used to manufacture knives, cutting tables and conveyor belts; (ii) cells within biofilms generally exhibit an increased resistance to cleaning and sanitization programs; and (iii) biofilms present on food-contact surfaces can act repeatedly as sources of contamination *(1, 2, 5)*. Further research is needed to determine what, if any, type of meat-contact surface material(s) is most suitable for use in constructing beef fabrication equipment and how to most effectively remove *E. coli* O157:H7 biofilms from such materials, especially in areas that may not be adequately cleaned.

Sanitizers, as defined by the Environmental Protection Agency (40CFR 455.10; *[4]*), are "intended to disinfect or sanitize, reducing or mitigating growth or development of microbiological organisms…on inanimate surfaces in the household, institutional, and/or commercial environment." Sanitizers are designed to inactivate microorganisms that survive and/or are translocated during the cleaning process. Several chlorine compounds, including sodium hypochlorite and acidified sodium chlorite, are approved for use as food-contact sanitizers (21CFR178.1010; *[3]*) and are generally effective against both gram-positive and gram-negative bacteria, although cells in biofilm tend to be less susceptible than planktonic cells *(23)*. Sodium hypochlorite is the most widely used commercial sanitizer, and efficacy increases when applied at a pH between 6.5 and 7.5 *(25)*. Decreased activity is observed in the presence of excess organic matter or hard water *(23)*. Chlorous acid, the active compound produced when sodium chlorite is combined with a generally recognized as safe (GRAS) weak acid (e.g., citric acid), exhibits an antimicrobial activity and spectrum similar to that of chlorine; its efficacy is optimal at pH ≤ 2.5 and in the absence of organic material *(8)*. A commercial solution of potassium peroxymonosulfate/sodium chloride, available for use as a broad spectrum hard surface disinfectant in livestock production and veterinary facilities, is not approved as a sanitizer for food-contact surfaces. In general, it is active against both gram-positive and gram-negative bacteria, and antimicrobial activity is markedly, but not entirely, reduced in the presence of excessive organic material or when mixed using hard water *(13)*.

Peroxyacetic acid is a powerful oxidizing agent approved for use on food-contact surfaces; its antimicrobial activity is optimal at elevated temperatures, at acidic pH values and in the absence of organic material. Antimicrobial activity is not affected by the presence of metallic ions (as in hard water sources) but is slightly diminished at low temperatures *(23)*. The U.S. Food and Drug Administration (FDA) has also approved a sanitizing solution that contains a mixture of peroxyacetic acid, acetic acid, octanoic acid, and hydrogen peroxide (21CFR178.1010; *[3]*).

Quaternary ammonium compounds are surface active agents (surfactants) approved for use on food-contact surfaces (21CFR178.1010; *[3]*); gram-negative bacteria are less susceptible to inactivation by such compounds than gram-positive bacteria *(23)*. Quaternary ammonium compounds are more effective when applied at higher temperatures (> 22°C, < 55°C) and at an acidic pH *(7)*. Activity is generally reduced in the presence of excessive organic material, metallic ions, anionic surfactants/detergents or soaps *(23)*. Cetylpyridinium chloride, a type of quaternary ammonium compound commonly used in oral hygiene products for its ability to impede attachment of plaque-forming bacteria to tooth enamel *(23)*, is approved for use as a poultry carcass decontamination fluid *(28)* but not for use on food-contact surfaces.

Sanitizers are designed to inactivate microbial contaminants present on clean surfaces, in the absence of organic material. Even so, the large size and complexity of commercial beef fabrication facilities, in conjunction with potentially inadequate employee training and/or guidance during cleanup, may contribute to improperly cleaned locations in difficult-to-reach parts of facilities and equipment. Therefore, although cleaning should always precede sanitation, it would be useful to identify sanitizers that might effectively reduce microbial contamination on both clean and soiled surfaces for use in such circumstances. For these reasons, this study was designed to investigate the effectiveness of seven sanitizing solutions in reducing numbers of *E. coli* O157:H7 biofilm cells on improperly cleaned food-contact surfaces, and the effect of surface material and biofilm age on sanitizer efficacy.

MATERIALS AND METHODS

Bacterial strains and inoculum preparation

The *E. coli* O157:H7 inoculum was comprised of six rifampicin-resistant derivatives of strains ATCC 51657, ATCC 51658, ATCC 43895, ATCC 43895/ISEHGFP *(14)*, and two strains recovered from beef cattle feces (F284, F469). Active cultures of individual strains were first prepared (35°C, 24 h) in 10 ml tryptic soy broth (Difco, Becton Dickinson, Sparks, MD) supplemented with rifampicin (100 µg/ml; Sigma-Aldrich, St. Louis, MO) and then subcultured under the same conditions. Cells of individual cultures were harvested by centrifugation (4,629 × g at 4°C for 15 min) (Eppendorf, 5810 R; Brinkmann Instruments, Inc., Westbury, NY) and then washed in 10 ml phosphate-buffered saline (PBS, pH 7.4; 0.2 g/liter KH_2PO_4, 1.5 g/liter $Na_2HPO_4 \cdot 7H_2O$, 8.0 g/liter NaCl, and 0.2 g/liter KCl). Washed cell pellets of each strain were resuspended in 10 ml PBS and combined to generate a 6-strain composite inoculum.

Inoculation of beef fat

The *E. coli* O157:H7 inoculum was used to inoculate beef fat, which in turn was used to inoculate the three types of beef fabrication surface materials evaluated in this study. This inoculation procedure simulated contamination of meat fabrication equipment surfaces with *E. coli* O157:H7 from pathogen-

TABLE 1. Sanitizers used and the concentrations evaluated in biofilm sanitizer challenges

Sanitizer	Active ingredient(s)	Manufacturers' recommended concentrations for use on food-contact surfaces	Sanitizer concentrations tested in this study	
			Minimum concentration (pH)	Maximum concentration (pH)
Acidified sodium chlorite (ASC)	sodium chlorite	500–1,200 ppm acidified to pH 2.5 with 20% sodium hydrogen sulfate solution	500 ppm (2.50)	1,200 ppm (2.50)
Sodium hypochlorite (SH)	8.5% sodium hypochlorite	100–200 ppm or 1–2 oz/6.5 gal	100 ppm (8.91 ± 0.33)	200 ppm (9.42 ± 0.23)
Potassium peroxymonosulfate (PP/SC)	20.4% potassium peroxymonosulfate 1.5% sodium chloride	Not an approved food-contact sanitizer	5,000 ppm (2.76 ± 0.09)	10,000 ppm (2.79 ± 0.11)
Quaternary ammonium chloride compounds (QACC)	3.0% alkyl dimethyl benzyl ammonium chloride 2.25% octyl decyl dimethyl ammonium chloride 1.35% didecyl dimethyl ammonium chloride 0.90% dioctyl dimethyl ammonium chloride	150–400 ppm or 1.25–2.67 oz/4 gal (based on water hardness)	200 ppm (6.77 ± 0.88)	400 ppm (6.81 ± 0.89)
Cetylpyridinium chloride (CPC)	40% cetylpyridinium chloride	Not an approved food-contact sanitizer	5,000 ppm (8.02 ± 0.33)	10,000 ppm (7.13 ± 0.61)
Peroxyacetic acid (PAA)	5.8% peroxyacetic acid 27.5% hydrogen peroxide	2,000–2,800 ppm	2,000 ppm (4.33 ± 0.44)	2,800 ppm (4.04 ± 0.45)
Peroxyacetic acid/octanoic acid mixture (PA/OA)	6.9% hydrogen peroxide 4.4% peroxyacetic acid 3.3% octanoic acid	1,300–2,600 ppm	1,300 ppm (4.43 ± 0.37)	2,600 ppm (3.98 ± 0.30)

contaminated beef carcass surfaces. Fresh beef fat purchased from a commercial beef processing facility was either used on the same day or stored (4°C) aerobically in plastic bags and used within 48 h. Beef fat was prepared for inoculation by trimming pieces to uniform thickness and laying them side by side on sterile foil-covered trays (external fat side up) to create a solid sheet of beef fat. The prepared *E. coli* O157:H7 inoculum (0.2 ml per 25 cm^2 sections) was uniformly spread onto the beef fat (pH 6.03), using a sterile bent glass rod, to achieve a target inoculum level of 6 log CFU/cm^2. Trays of inoculated beef fat were then covered and stored at 4°C for 24 h to simulate beef carcass chilling.

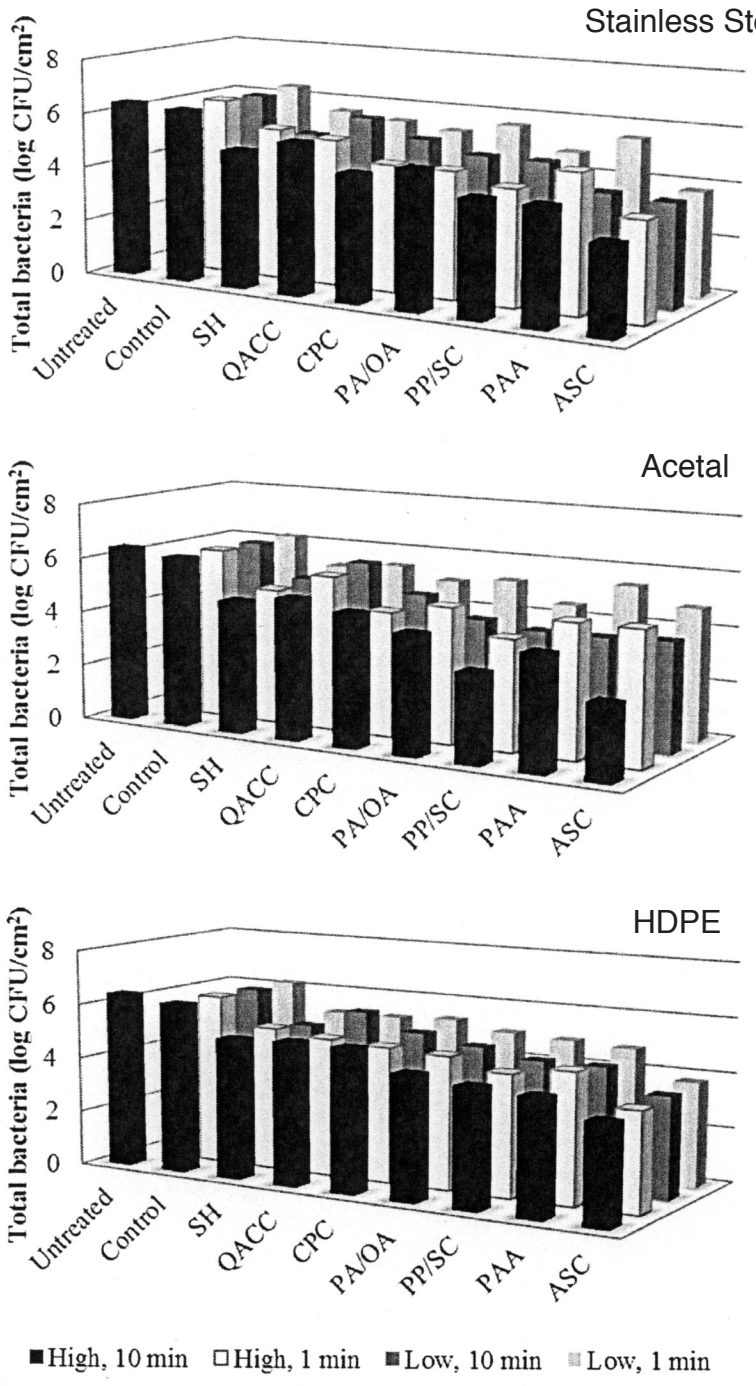
Inoculation and storage of surface materials

The materials most commonly used to construct meat fabrication equipment were identified as stainless steel (type 304, #2b finish), acetal and HDPE. Before use, coupons (2 × 5 cm) of each material were washed with dish soap and water to remove any processing residue, followed by rinsing with water, soaking in 70% ethyl alcohol, rinsing with water, air-drying, and sterilization by autoclaving. Dry, sterile coupons of each material were placed between two pieces of inoculated beef fat and held (4°C) under pressure (20 kg, to simulate the pressure that would be applied to meat-contact surfaces by carcass primals and subprimals) for 30 min. Inoculation of both sides of each coupon was required, as surfaces were to be submerged in the incubation substrate for biofilm formation and subsequently treated with the sanitizing treatments by immersion in the solution (described below). After inoculation, coupons were rinsed with 20 ml sterile distilled water, to remove unattached or loosely attached cells, and were partially submerged *(9)* in sterile polypropylene tubes (50 ml, Fisher Scientific, Fair Lawn, NJ) containing 20 ml of fresh, unsterilized beef fat-lean tissue homogenate (FLH). The FLH was prepared by pummeling (2 min; Masticator, IUL Instruments, Barcelona, Spain) equal portions (1:1 wt/wt) of beef fat and lean tissue with sterile distilled water to yield a 10% (wt/wt) suspension that was then passed through sterile cheesecloth to obtain a liquid portion to be used as the incubation substrate for the inoculated coupons. The presence of organic material and the nutrient and microbial composition of the incubation substrate thus represented liquid residues likely to be encountered in pre-sanitation and/or ineffectively cleaned and sanitized beef fabrication environments. The tubes containing the surface materials were incubated at 15°C for up to 7 days.

Sanitizer treatment

After 3 days of incubation, coupons of each material were removed from the incubation substrate, rinsed with 20 ml of sterile distilled water (to remove unattached or loosely attached cells) and then placed in 40 ml of sterile distilled water (control) or sanitizing solution for 1 or 10 min. Submersion was selected instead of spray applications to expose all sides of the coupons evenly to sanitizing solutions and to eliminate the potentially added effects of spray nozzle pressure. The sanitizers used were commercially-available products or were prepared in the laboratory to simulate other commercially-available products. Seven sanitizers (Table 1) were tested: acidified sodium chlorite (generated in the laboratory; ASC), a commercial sodium hypochlorite solution (SH; XY-12, Ecolab, St. Paul, MN), a commercial potassium peroxymonosulfate/sodium chloride solution (PP/SC; Virkon® S, DuPont, Wilmington, DE), a commercial mixture of quaternary ammonium chloride compounds (QACC; Oasis™, Ecolab), a commercial cetylpyridinium chloride solution (CPC; Cecure™, Safe Foods Corporation, North Little Rock, AR), a commercial peroxyacetic acid solution (PAA; Oxonia Active™, Ecolab), and a commercial peroxyacetic acid/octanoic acid mixture (PA/OA; Vortexx®, Eco-

FIGURE 1. Surviving total aerobic bacteria (log CFU/cm^2), recovered with tryptic soy agar, attached to stainless steel, acetal or high-density polyethylene (HDPE) coupons (2 × 5 cm) after 3 days incubation (15°C) in fresh beef fat-lean tissue homogenate (untreated), and after subsequent treatment (1 or 10 min) with distilled water (control) or sanitizing solutions applied at minimum (low) or maximum (high) recommended concentrations. ASC: acidified sodium chlorite; SH: sodium hypochlorite; QACC: a mixture of quaternary ammonium chloride compounds; CPC: cetylpyridinium chloride; PAA: peroxyacetic acid; PA/OA: a peroxyacetic acid/octanoic acid mixture; PP/SC: potassium peroxymonosulfate/sodium chloride.



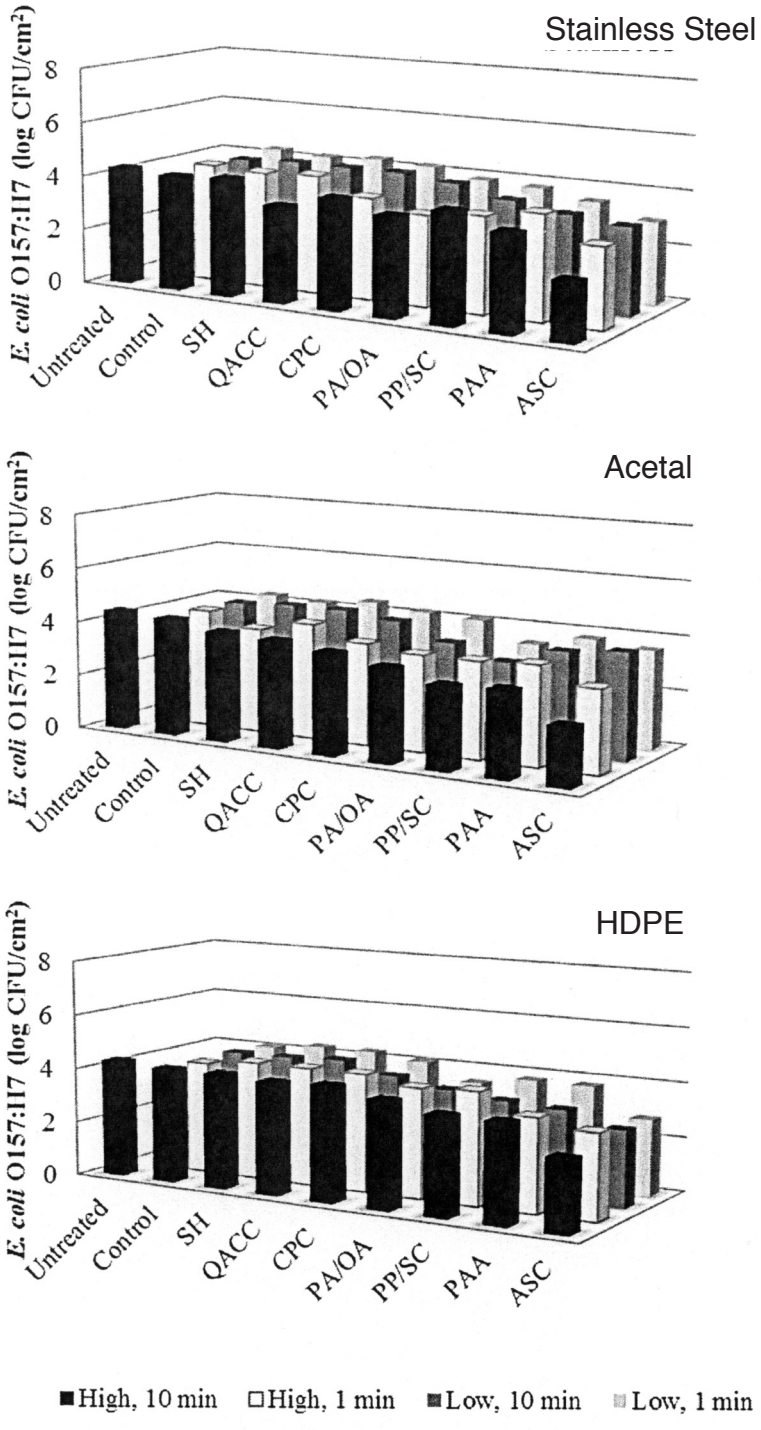
lab). These sanitizing solutions included those most commonly used in the food industry as well as some not currently approved for use as food-contact surface sanitizers (Table 1). All sanitizing solutions were mixed and stored as directed by the manufacturer and were applied at the minimum and maximum concentrations recommended by the manufacturer (Table 1). The ASC solution was prepared by first mixing sodium chlorite (Sigma-Aldrich) in sterile distilled water to generate 500 ppm and 1,200 ppm stock solutions which were then sealed and stored at room temperature for up to 7 days before use. Aliquots of aqueous sodium chlorite stock solutions were slowly mixed with 20% aqueous sodium hydrogen sulfate (Sigma-Aldrich; 1:5 wt/wt in sterile distilled water) until a final pH of 2.5 was achieved and maintained for 3 min. The resulting ASC solution was used immediately, and fresh solutions were prepared for each treatment.

In a separate experiment, conducted to evaluate the effect of biofilm age on efficacy of sanitizing treatments, three of the seven sanitizers (SH, QACC, PA/OA) and a sterile distilled water control were applied (1 or 10 min, at manufacturer-recommended minimum and maximum concentrations; Table 1) to inoculated coupons (as previously described) incubated at 15°C in the FLH for 0 (12 h), 3 and 7 days.

Microbiological and pH analyses

Following inoculation, initial (day 0 [12 h]) microbial populations on coupon surfaces (three per treatment in each of two biologically independent experiments) were determined. The coupon surfaces were first rinsed with 20 ml sterile distilled water, to remove unattached/loosely attached cells, and then placed in tubes containing 40 ml diluent (comprised of 0.85% aqueous NaCl solution [Fisher] and 0.1% peptone [Difco]) and 10 glass beads. To enumerate microbial populations on coupon surfaces before and after sanitizer treatments, coupons (three per treatment in each of two biologically independent experiments) were placed in tubes containing 40 ml D/E neutralizing broth (Difco) and 10 glass beads. All samples were then vortexed for 2 min (3,200 rpm), serially diluted in 0.1% buffered peptone water

FIGURE 2. Surviving inoculated *E. coli* O157:H7 populations (log CFU/cm^2), recovered with tryptic soy agar plus rifampicin (100 µg/ml), attached to stainless steel, acetal or high-density polyethylene (HDPE) coupons (2 × 5 cm) after 3 days incubation (15°C) in fresh beef fat-lean tissue homogenate (untreated), and after subsequent treatment (1 or 10 min) with distilled water (control) or sanitizing solutions applied at minimum (low) or maximum (high) recommended concentrations. ASC: acidified sodium chlorite; SH: sodium hypochlorite; QACC: a mixture of quaternary ammonium chloride compounds; CPC: cetylpyridinium chloride; PAA: peroxyacetic acid; PA/OA: a peroxyacetic acid/octanoic acid mixture; PP/SC: potassium peroxymonosulfate/sodium chloride.



(Difco) and surface-plated onto tryptic soy agar (TSA; Difco) for enumeration of total bacterial populations, and onto TSA supplemented with rifampicin (100 µg/ml; TSArif) for enumeration of inoculated *E. coli* O157:H7 populations. Uninoculated beef fat samples were also analyzed to determine the natural microbial contamination level. Plates were incubated at 25°C for 48 h (TSA) or 35°C for 24 h (TSArif), after which colonies were enumerated. The pH of inoculation and incubation substrates, on each analysis day, as well as of all sanitizer solutions, was measured by use of a digital pH meter fitted with a glass electrode (Denver Instruments, Arvada, CO).

Statistical analysis

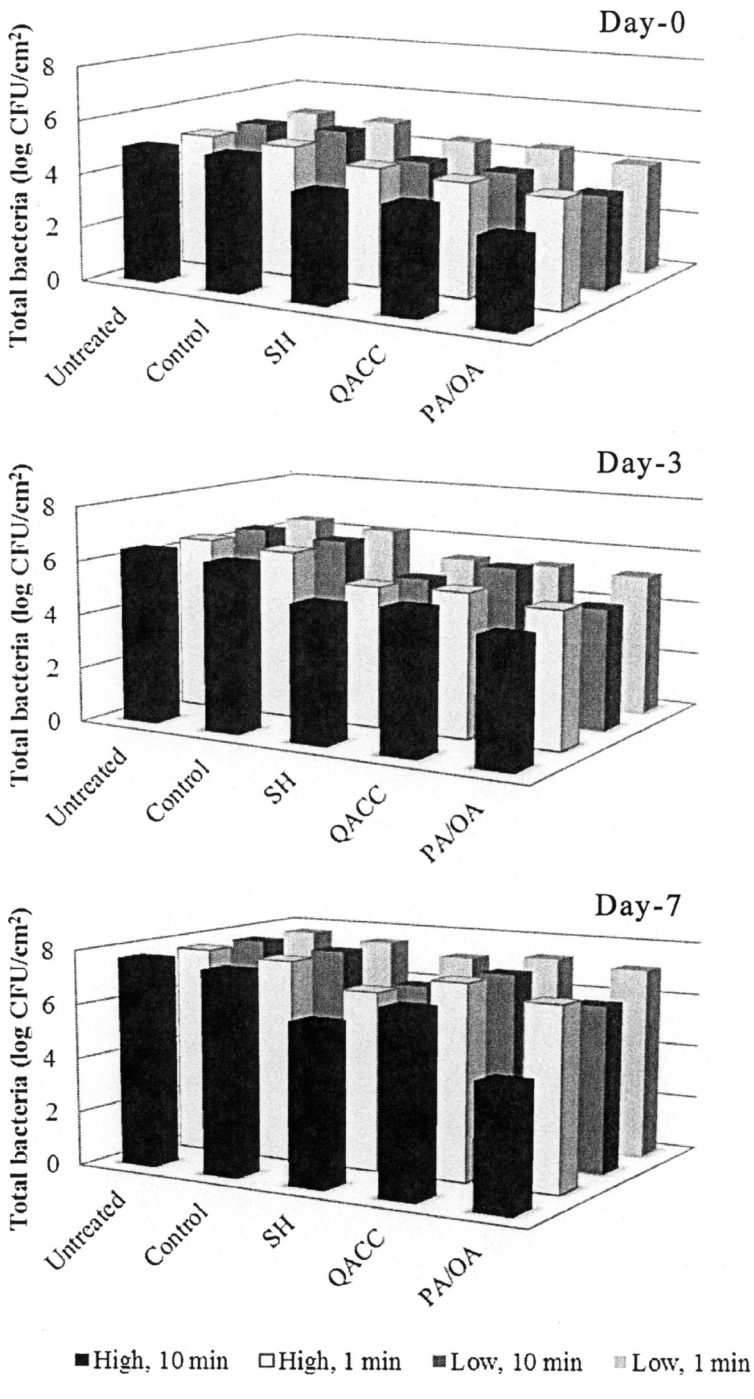
Microbiological data were converted to log CFU/cm^2 and least squares means were calculated using the analysis of variance in the general linear model procedure of SAS (version 9.1, SAS Institute, Cary, NC). Under the conditions of this study, the detection limit of the microbial analysis was 0.30 log CFU/cm^2. All samples with microbial counts below the detection limit were assigned a value of 0.29 log CFU/cm^2. Treatment effects included surface material (stainless steel, acetal or HDPE), sanitizer (water control, ASC, SH, PP/SC, QACC, CPC, PAA, or PA/OA), sanitizer concentration (Table 1), sanitizer treatment exposure time (1 or 10 min) and incubation period or age of biofilm (0, 3 or 7 days). All treatment effects were analyzed individually and as interactions, and *P*-values less than 0.05 ($P < 0.05$) were considered statistically significant.

RESULTS AND DISCUSSION

Initial cell attachment and biofilm formation

Unpublished data from our laboratory have indicated that exposure to *E. coli* O157:H7-inoculated beef fat was a successful and realistic means of transferring microbial contamination onto meat-contact surface materials. The outer surface of a beef carcass is comprised almost entirely of subcutaneous fat that acts as the primary site of microbial contamination during carcass dressing. Thus, the contaminated subcutaneous beef fat used in this study is the most logical vehicle for transferring contamination onto breaking

FIGURE 3. Surviving total aerobic bacteria (log CFU/cm^2), recovered with tryptic soy agar, attached to coupons (2 × 5 cm) after 0, 3 and 7 days incubation (15°C) in fresh beef fat-lean tissue homogenate (untreated), and after subsequent treatment (1 or 10 min) with distilled water (control) or sanitizing solutions applied at minimum (low) or maximum (high) recommended concentrations. SH: sodium hypochlorite; QACC: a mixture of quaternary ammonium chloride compounds PA/OA: a peroxyacetic acid/octanoic acid mixture.



surfaces and utensils. The natural microbial contamination level of the uninoculated beef fat samples analyzed was 2.8 ± 0.6 log CFU/cm^2, as recovered on TSA, and no rifampicin-resistant *E. coli* O157:H7 populations were recovered (detection limit was 0.3 log CFU/cm^2) on TSArif (data not shown). Based on additional unpublished research from our laboratory, maximal *E. coli* O157:H7 biofilm formation was observed between 2 and 8 days of incubation at 15°C (the average temperature of commercial beef processing facilities during non-production hours) and was dependent on the presence/absence of an air-liquid interface *(9)* and characteristics of the incubation substrate. For these reasons, in this study, stainless steel, acetal and HDPE coupons were exposed to inoculated beef fat and then partially submerged in FLH and stored for up to 7 days at 15°C. The pH of the FLH was 5.60 on day 0 and was similar (5.49 ± 0.28) throughout storage. On days 0, 3 and 7 of storage, total aerobic bacteria counts recovered from coupon surfaces were 5.0 ± 0.2, 6.4 ± 0.7 and 7.7 ± 0.3 log CFU/cm^2, respectively, and corresponding *E. coli* O157:H7 counts were 3.6 ± 0.4, 4.3 ± 0.6 and 3.7 ± 0.5 log CFU/cm^2, respectively.

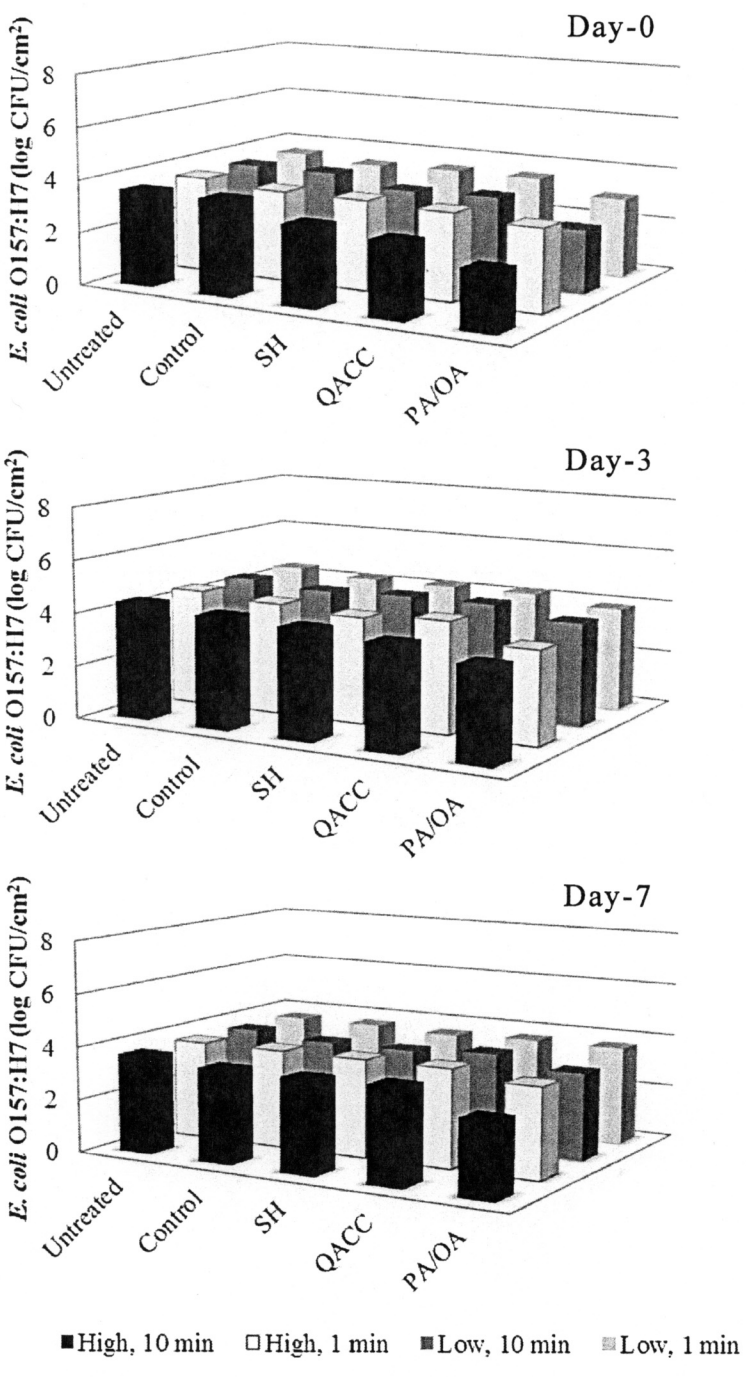
Effect of surface material on sanitizer-induced inactivation of biofilm cells

Overall, there were no significant ($P \geq 0.05$) differences in sanitizer-associated microbial reductions among the three surface materials when the efficacy of the seven sanitizers was evaluated against biofilm cells at 3 days of incubation (Fig. 1 and 2) or when three selected sanitizers (SH, QACC, PA/OA) were evaluated against biofilm or attached cells at 0 (12 h), 3 or 7 days of incubation (data not shown). When differences in reductions of *E. coli* O157:H7 counts were observed among the material types, the larger reductions were associated with populations on acetal coupons, followed by HDPE and then stainless steel (Fig. 1 and 2).

Effect of sanitizer type, concentration and exposure time on inactivation of biofilm cells

A significant ($P < 0.05$) three-way interaction was obtained between sanitizer type, sanitizer concentration and sanitizer treatment exposure time. Sanitizer treatments reduced total aerobic populations (6.4 ± 0.7 log CFU/cm^2, on day 3 of incubation) on coupon surfaces by 0.6 to 3.7 log CFU/cm^2, and sanitizer efficacy decreased in order of ASC > PAA > PP/SC > PA/OA > CPC > QACC = SH > control (Fig. 1). Efficacy against inoculated *E. coli* O157:H7 populations (4.3 ± 0.6 log CFU/cm^2 on day 3 of incubation) attached to coupon surfaces

FIGURE 4. Surviving inoculated *E. coli* O157:H7 populations (log CFU/cm^2), recovered with tryptic soy agar plus rifampicin (100 µg/ml), attached to coupons (2 × 5 cm) after 0, 3 and 7 days incubation (15°C) in fresh beef fat-lean tissue homogenate (untreated), and after subsequent treatment (1 or 10 min) with distilled water (control) or sanitizing solutions applied at minimum (low) or maximum (high) recommended concentrations. SH: sodium hypochlorite; QACC: a mixture of quaternary ammonium chloride compounds PA/OA: a peroxyacetic acid/octanoic acid mixture.



decreased in order of ASC > PAA > PP/SC = PA/OA > CPC > QACC = SH = control, with sanitizer-induced reductions ranging from 0.0 to 2.2 log CFU/cm^2 (Fig. 2). Microbial reductions generally increased as sanitizer concentration and exposure time increased, although ASC treatments were more effective ($P < 0.05$) against 3-day-old *E. coli* O157:H7 biofilm cells than any other sanitizing solution, regardless of concentration/exposure time combination (Fig. 2). The fate of biofilm cells in response to SH and ASC sanitizing treatments differed, even though both solutions were chlorine-based. Sodium hypochlorite is easily inactivated in the presence of organic material and, as an oxidizing agent, must interact with cell surfaces to be effective *(23)*. Furthermore, the 100 and 200 ppm SH solutions used in this study were mixed according to the manufacturers' recommendations and had a pH of 8.91 and 9.42, respectively (Table 1). The antimicrobial activity of SH solutions is optimal at a pH of 6.5 to 7.5 *(24, 25)*, and greater activity against *E. coli* O157:H7 may have been observed in this study if the SH solutions had been adjusted to a pH of ≤ 7.5 *(24)*. No recommendations were, however, given by the manufacturer to make any adjustments to the pH of the SH solutions. Chlorous acid, the primary active agent in ASC solutions, is an oxidizing agent and is therefore sensitive to organic material. However, ASC does possess more oxidizing potential than chlorine *(8)*, which, in combination with a very low solution pH (pH 2.5), may explain the greater efficacy of ASC than of SH in this study. Given that PP/SC (10,000 ppm) also reduced total aerobic bacteria and *E. coli* O157:H7 on soiled surfaces, additional research may be needed to determine whether its use on food-contact surfaces is appropriate.

Sanitizer-induced inactivation of microbial populations as affected by age of biofilms

Three of the compounds most commonly used to sanitize commercial beef fabrication facilities, identified via personal communication with chemical sales representatives and sanitation professionals, are SH, QACC and PA/OA. To determine the influence of biofilm age on sanitizer efficacy, four factors were evaluated: sanitizer type, sanitizer concentration, sanitizer treatment exposure time, and age of biofilm cells; this resulted in a four-way interaction ($P < 0.05$). On days 0, 3 and 7, sanitizer treatments reduced initial total aerobic bacteria populations on coupon surfaces (5.0, 6.4 and 7.7 log CFU/cm^2, respectively) by 0.6 to 1.8, 0.7 to 1.9 and 0.6 to 3.2 log CFU/cm^2, respectively (Fig.

3). Sanitizing treatments reduced initial *E. coli* O157:H7 populations on days 0, 3 and 7 (3.6, 4.3 and 3.7 log CFU/cm^2, respectively) by 0.1 to 1.4, 0.1 to 0.8 and 0.0 to 0.9 log CFU/cm^2, respectively (Fig. 4). *E. coli* O157:H7 counts were reduced by 0.0 to 0.2 log CFU/cm^2 by the water control treatment, irrespective of biofilm age (Fig. 4). Microbial reductions generally increased as sanitizer concentration and exposure time increased (Fig. 3 and 4). PA/OA treatments were more effective ($P < 0.05$) than QACC or SH treatments against total aerobic bacteria and *E. coli* O157:H7 regardless of biofilm age. QACC treatments were effective against newly established biofilms but ineffective against older biofilm cells (Fig. 4). SH treatments were effective against total aerobic bacteria but ineffective against *E. coli* O157:H7 biofilm cells, regardless of biofilm age, sanitizer concentration or exposure time. Quaternary ammonium chloride compounds and sodium hypochlorite, which are among the most commonly used commercial sanitizers, are generally effective when applied to clean soil-free surfaces *(7, 11)*. In a study by Lomander et al. *(12)*, food-contact-approved concentrations (200 ppm) of sodium hypochlorite effectively detached *E. coli* biofilms from the surface of polished stainless steel but not from scratched stainless steel. In another study *(19)*, sodium hypochlorite was found more effective than peracetic acid solutions in inactivating *E. coli* on stainless steel. These studies evaluated sanitizer efficacy against cells on clean surfaces, whereas the present study evaluated efficacy in the presence of organic soil. As previously indicated, a limitation of using SH as a sanitizing solution is its sensitivity to organic compounds *(7)*; thus, its inability to inactivate the pathogen under the conditions of this study was not unexpected. Quaternary ammonium compounds are also sensitive to organic material, but to a lesser degree than chlorine, and also possess surfactant properties *(7)*. Uhlich et al. *(27)* found that 1 or 2 min exposures to quaternary ammonium sanitizer (1:64 vol/vol dilution) were ineffective against 48 h-old *E. coli* O157:H7 biofilms (7.5 log CFU/ml) on glass, Teflon or stainless steel coupons, while 10 min exposures to 5.0% hydrogen peroxide reduced biofilm populations by 1 to 4 log CFU/ml. The PA/OA solution used in the current study was comprised of multiple active ingredients, including hydrogen peroxide, peroxyacetic acid, and octanoic acid, which may in part explain the greater activity of PA/OA solutions than of QACC or SH solutions against *E. coli* O157:H7 biofilms cells.

Overall, susceptibility of biofilm-associated total aerobic populations to sanitizer treatments did not change as age of biofilm increased (Fig. 3). In contrast, *E. coli* O157:H7 biofilm cells became less sensitive to most sanitizer treatments as age of the biofilm increased (Fig. 4). In general, 1 min exposures to sanitizing solutions were ineffective against *E. coli* O157:H7 biofilm cells, regardless of biofilm age, type of sanitizer or sanitizer concentration (Fig. 4). Efficacy of 10 min exposures against *E. coli* O157:H7 biofilm cells decreased ($P \geq 0.05$) as biofilm age increased, and with the exception of maximum recommended concentrations of PA/OA for 10 min exposures, no concentration/exposure time combination of SH, QACC or PA/OA was capable of reducing *E. coli* O157:H7 populations in 7-day-old biofilms by more than 0.5 log CFU/cm^2 (Fig. 4). Although exposure to maximum concentration sanitizer treatments typically resulted in equivalent or greater microbial reductions than minimum concentration treatments, differences in reductions between minimum vs. maximum recommended concentration treatments were not consistently significant, regardless of biofilm age (Fig. 3 and 4).

The increased resistance of biofilm versus planktonic cells to sanitizing treatments is well established *(1, 5, 24)*. Although not all aspects of increased resistance are fully understood, it is generally recognized that cells attached to surfaces have less available surface area than planktonic cells for antimicrobial-cell interaction and that the glycoproteins, exopolysaccharides and other compounds (glycocalyx) surrounding biofilm cells also act to protect cells physically from surface-active agents *(27)*. For these same reasons, mature biofilms with thicker glycocalyx layers should be more resistant to sanitizing treatments than newly established biofilms *(15, 20)*.

In summary, commercial beef processing facilities are usually large and house multiple types of equipment, which require various degrees of disassembly and attention during cleaning and sanitation processes and whose surfaces, at the end of a production shift, are typically covered by a heavy layer of beef fat and lean tissue. Cleaning protocols that employ both chemical and physical removal of organic material from these surfaces are critical to the success of ensuing sanitizer treatments. Even so, removal of all organic material from the entirety of every surface in a large and complex facility may not be complete, even after intensive cleaning efforts, especially in hard-to-reach locations. Small amounts of organic material may be left behind in crevices or difficult-to-clean parts. It is also important to note that all surface materials used in this study were in good condition and free of visible surface damage. It is generally accepted that even microscopic nicks and cuts on surfaces can harbor microbial contaminants. Therefore, although new surface materials did not appear to directly influence sanitizer efficacy, care should be taken to select materials that can withstand the daily wear and tear associated with manually boning beef and that do not chip or feather easily, as rough or scratched surfaces are increasingly difficult to clean and sanitize successfully *(6, 12)*.

Under the conditions of this study, sanitizer-induced pathogen reductions on soiled surfaces were small (≤ 2.2 log CFU/cm^2), increased as sanitizer concentration and exposure time increased, and decreased as biofilm cells aged. It is well known that sanitizers alone cannot compensate for inadequate cleaning protocols and that the removal of organic material from surfaces is essential to effective sanitation. It is also apparent that care should be taken to apply sanitizers at the highest allowable concentration for extended dwell times. Concentrations of acidified sodium chlorite and peroxyacetic acid-based sanitizers that are approved for use on food-contact surfaces were effective against total aerobic bacteria and *E. coli* O157:H7 biofilm cells on soiled surfaces, and their systematic use should be considered where there is a need to address contamination in hard-to-clean places. Furthermore, quaternary ammonium chloride compounds and sodium hypochlorite, the two sanitizing chemicals most commonly used in the food manufacturing industry, were the least effective against *E. coli* O157:H7

biofilm cells on soiled surfaces, regardless of biofilm age, sanitizer concentration or exposure time. Lastly, processors may consider weekly applications of sanitizing solutions that exceed approved levels for food-contact surface sanitation, although food-contact surfaces treated with such solutions would have to be rinsed with water to avoid corrosion and before operations are allowed to resume. Such treatments would require an additional step but could help negate the establishment of microbial biofilms.

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